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2. Patent application number *(The Patent Office will fill in this part)* — 3 AUG 2002

3. 0218089.1

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GRAFTON STREET
MANCHESTER
M13 9XX
UNITED KINGDOM

Patents ADP number *(if you know it)* — 8401077001

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention — TARGET DNA

5. Name of your agent *(if you have one)* — Marks & Clerk

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TARGET DNA.

The present invention relates to collections of labelled target DNA.

Recent years have seen a growth in the realisation of the importance of gene expression in the control of biological activities. It is known that expression of specific subsets of genes regulate tissue formation and organogenesis during development and also the properties of adult tissues. Patterns of gene expression influence not only the structure and composition of specific tissues, but also the tissues' responses to various stimuli. These structures, composition and responses, and the patterns of gene expression encoding them, are distinctive markers for individual tissues.

At a more complex level the pattern of genes expressed by whole organisms may be characteristic of specific individuals and provide an insight into their biological status. For instance, there is growing evidence that the pattern of genes expressed by an individual may influence factors such as the individual's predisposition to particular diseases or their responsiveness to certain therapeutic agents.

The current challenge to biologists is to learn how the products of the around 40,000 identified human genes interact to produce the complexity exhibited by higher eukaryotes. To a large extent the biological character of a cell can be inferred from the profile of genes it expresses. Although an examination of mRNA or protein expression patterns alone does not directly address function, the knowledge of when and where a gene is expressed can provide valuable insights as to the potential role of a gene and has historically been instrumental in the discovery of developmentally regulated genes. Recognition of the value of the examination of expression patterns led to the development of a plethora of advanced mRNA profiling technologies such as cDNA microarrays (Duggan et al., 1999), SAGE (Velculescu et al., 1995), and cDNA display (Liang and Pardee, 1992) aimed at the simultaneous measurement of tens to several thousand genes in the target samples. Application of these profiling technologies to clinical diseases, such as cancer has confirmed the utility of profiling and provided useful diagnostic and prognostic assays (Shipp et al., 2002; Staunton et al., 2001; van 't Veer et al., 2002).

Despite the success of these approaches at the molecular level by identifying patterns of expression exhibited generally by relatively homogeneous cellular samples the cellular complexity of higher eukaryotes still presents a major obstacle to expression profiling.

Over the last 30 years a variety of molecular techniques have been developed for the analysis of gene-expression. In general methods focussed either on the identification and characterisation of genes (either individual genes or networks of related genes) or the characterisation of the input tissue or cell based on a characteristic profile of expressed genes. Although conventional nucleic acid hybridization techniques (such as northern and dot blots) have been used for many years to analyse a small number of genes and samples there have been a variety of advanced mRNA profiling technologies such as cDNA microarrays (Duggan et al., 1999), SAGE (Velculescu et al., 1995), and cDNA display (Liang and Pardee, 1992) which have been recently developed to allow the simultaneous measurement of tens to several thousand genes in the target samples.

Many of the techniques for analysis of gene-expression described above require the use of labelled target DNA capable of binding to complementary DNA sequences in reference samples. In order to take both full advantage of and to extend recent improvements in gene-expression analysis it is important that the labelled target DNA be sensitive, that is to say having a high binding affinity for complementary DNA sequences. It is also beneficial to be able to produce labelled target DNA from small samples, ideally single cells, since this allows a greater range of cell types to be used (since it obviates a requirement for large numbers of cells), and improves confidence that the starting population is "pure", rather than representing a mixed population of cell types such as is found in many tissue samples. Furthermore, it is advantageous if labelled target DNA can be produced rapidly, by cheap simple techniques. Unfortunately many known collections of labelled target DNA suffer from disadvantages in that they have relatively low sensitivity, or are prepared by laborious, complicated or expensive techniques.

It is an object of the present invention to obviate or mitigate the disadvantages associated with the prior art.

According to the present invention there is provided a collection of labelled target DNA molecules which are exonuclease derivatives of cDNA.

Collections of labelled target DNA molecules according to the invention provide a number of advantages over prior art target DNA collections, as set out below. Briefly, target DNA collections of the invention provide advantages in terms of their enhanced sensitivity, their ability to be prepared from small samples, and their ease and cost of preparation.

Collections of labelled target DNA molecules according to the invention have greater sensitivity than previously described targets since the single-stranded target DNA molecules of the invention are not susceptible to "self-hybridisation". Thus collections of labelled target DNA according to the invention, when used in a hybridisation-based assay, are more readily able to hybridise with complementary DNA sequences in a reference sample, should such sequences be present. Furthermore, preparation of the target molecules is more flexible, cheaper and simpler than prior art techniques. These advantages arise from the fact that the collection of target molecules can be prepared from small amounts of starting material (thereby avoiding costly purification steps and increasing the variety of samples from which labelled target DNA can be prepared), and can be prepared using cheap, simple techniques. Furthermore, since labelled target DNA of the invention can be prepared from samples as small as a single cell, it is possible to ensure that the starting population from which the target DNA is prepared represents a pure population as opposed to a mixture of different cell types.

The collection of labelled target DNA molecules may be prepared by a number of different methods. The methods described below are simple allowing easy, cost-effective preparation of the collections of labelled target DNA.

One method suitable for the preparation of a collection of labelled target DNA molecules according to the invention is to subject cDNA or a derivative thereof (e.g. a DNA population produced by total or partial amplification of the cDNA population) to

exonuclease digestion such that a collection of essentially single-stranded DNA molecules is produced, and then to label these single-stranded molecules.

Conveniently the single-stranded molecules may be labelled by incorporation of labelled nucleotides at the 3' end of the single-stranded DNA molecules using the template-independent DNA polymerase terminal transferase.

An alternative method by which collections of the invention may be prepared is to treat double-stranded cDNA or a derivative thereof to obtain a labelled double-stranded DNA population and then to effect exonuclease digestion of the labelled DNA population. Production of labelled double-stranded DNA from the double-stranded cDNA population (or derivative) may, for example, be effected by addition of labelled nucleotides via the DNA polymerase terminal transferase (as described above). Alternatively a labelled double-stranded DNA population may be derived from the cDNA or derivative through amplification of the original cDNA by well-known polymerase chain reaction (PCR) techniques using labelled nucleotides. The labelled double-stranded DNA population may then be subjected to exonuclease digest in order to produce a substantially single-stranded labelled DNA population. In cases in which the label is incorporated using PCR this provides an advantage in that the efficiency of label incorporation can be readily assessed by gel electrophoresis and/or real-time quantitative PCR.

Conveniently labelled double-stranded DNA representative of gene expression in a sample of interest may be prepared using primers comprising a homopolymer T tract (for example CATCTCGAGCGGCCGCTTTTTTTTTTTTTTTTTTTTTTT). An example of this amplification technique is described in Brady et al. (1990). When combined with homopolymer tailing (for example using terminal transferase) PCR using such primers produces a population of DNA molecules, in which all molecules have a poly-T region at one end and a poly-A region at the other. This technique has the advantage that a single oligonucleotide can be used for the initial and all subsequent PCR amplifications. The technique also obviates the need to create new priming sites within the molecules to be amplified, since each molecule produced by amplification contains a poly-A region that can anneal to a poly-T region in the primer allowing further rounds of amplification.

Primers comprising a poly-T tail (as described above) may also comprise a further sequence of nucleotides in addition to the tail region. Such further nucleotides may be selected to allow the incorporation into DNA molecules, produced by PCR using these primers, of regions that may be advantageous for the further amplification or subsequent use of molecules produced. For example primers may be designed such that they will incorporate "anchor" sequences (thereby enabling improved specificity of subsequent PCR) or cloning sites (allowing subsequent manipulation of amplified DNA products). Suitable sequences for incorporation into such primers to achieve these purposes would be immediately appreciated by one skilled in the art.

It will be appreciated that the methods described above may be effected by the use of a kit according to appropriate instructions. Accordingly there is provided a kit for the preparation of a collection of labelled target DNA molecules according to the invention, the kit comprising:

(i) an exonuclease;
(ii) terminal transferase; and
(iii) labelled nucleotides.

There is also provided a kit for the preparation of a collection of labelled target DNA molecules according to the invention, the kit comprising:

(i) an exonuclease;
(ii) primers; and
(iii) labelled nucleotides.

A collection of target DNA molecules according to the invention may be labelled by incorporation of labelled nucleotides within the DNA molecules. Labelled nucleotides may incorporate a detectable moiety, or may contain a functional group (e.g. an amino group) that is subsequently able to react with a detectable moiety. Suitable detectable moieties include fluorescent moieties (flurophores), radio-labelled moieties, and enzymes capable of producing a chromogenic reaction with a suitable substrate. In a preferred embodiment DNA molecules according to the invention are directly labelled by

incorporation of nucleotides labelled with fluorescent moieties. This technique provides the advantage that relatively small quantities of fluorescent label are required. This has obvious benefits in terms of reducing the cost associated with the production of labelled target DNA. Suitable examples of commercially available fluorescently labelled nucleotides include FluoroLink nucleotides, which are supplied by Amersham Pharmacia Biotech.

In a preferred embodiment of the invention the cDNA from which the labelled single-stranded DNA population is derived is globally amplified cDNA. By globally amplified cDNA we mean cDNA in which DNA molecules representing gene expression retain the same relative abundance as the mRNA transcripts from which they are derived.

There are a number of known techniques by which globally amplified cDNA suitable for use in the invention may be produced. Most preferably the global amplified cDNA is prepared from mRNA using limiting concentrations of nucleotides and a relatively short incubation time in order to limit cDNA synthesis. This ensures that, no matter what the length of the original mRNA transcript, all cDNA molecules produced are of approximately the same relatively small size. Since all the cDNA molecules are of approximately equal size subsequent amplification of the cDNA results in equal reproduction of all the cDNA molecules present. This ensures that the amplified cDNA produced reflects the original relative abundance of the mRNA present in the biological sample. Suitable protocols for the production of global amplified cDNA of this nature are provided in Brady *et al.* 1990, Cumano et al. 1992 and Brady *et al.* 1993. In addition to the advantage of allowing the production of amplified populations of cDNA that maintain the relative abundance of the original mRNA the use of global amplified cDNA also provides other advantages. For example global amplified cDNA can be derived either directly from one or more freshly isolated living cells without the need for RNA isolation, or from mRNA purified from a biological sample. Additionally, the production of global cDNA is well suited to automation, providing advantages in terms of ease and speed of use.

The use of globally amplified cDNA in the production of collections of labelled target DNA according to the invention provides a number of advantages. A first advantage arises from the fact that globally amplified cDNA can be produced from samples as small as a single cell, which may typically contain in the region of 20pg total RNA. Since conventional techniques for the production of collections of target DNA typically require starting quantities of RNA in the region of 20 μg the ability to work with single cells represents a million fold increase template sensitivity. A second advantage of the use of globally amplified cDNA is that large amounts of DNA can be made, which can be readily and simply checked by methods such as gel electrophoresis and/or real-time quantitative PCR prior to and/or following incorporation of label. This provides advantages not only in terms of ease of production, but also in that it avoids the costs associated with inefficient labelling of target DNA molecules and ineffective use or wastage of arrays.

Exonuclease digestion to produce collections of target DNA according to the invention may be performed using a suitable 3' or 5' exonuclease.

Treatment of double-stranded DNA molecules (from which the collection of target molecules is derived) with exonuclease results in degradation from either the 3' or 5' end of each strand of DNA (depending on the specificity of the enzyme selected). Thus regions of each strand that are complementary to one another are removed by digestion. Digestion with double-stranded DNA (dsDNA) exonucleases will initiate digestion at each end of a double-stranded DNA molecule. Since the dsDNA exonuclease preferentially removes one strand of the double-stranded molecule digestion tends to be "self-limiting", and will decrease when there are no remaining regions of double-stranded DNA. Thus the exonuclease treatment can effectively convert each starting double-stranded DNA molecule into two non-complementary single-stranded DNA molecules corresponding to the 3' or 5' "halves" of the original molecule.

Alternatively with knowledge of the average size of molecules within the double-stranded DNA population (determined, for example, by gel electrophoresis) and the rate of digestion by the chosen exonuclease it is possible to chose an incubation period such that

the digestion removes a chosen length of the DNA molecules. This chosen length may, for example, be approximately half the average DNA molecule length present in the starting double-stranded DNA population. Such a digest will, as with the technique described above, produce two single-stranded DNA molecules corresponding to the (3' or 5') "halves" of the lengths of the two starting strands of the original double-stranded DNA.

It will be appreciated that as a result of the digestion the two remaining molecules are not complementary to one another. This therefore prevents the strands of target DNA re-hybridising to their complementary sequences found within the original double-stranded DNA population. Thus the collection of target DNA molecules are maintained in single stranded form and are therefore free to hybridise to complementary single-stranded DNA sequences in a reference sample to which they are exposed (should such sequences be present). This improves the sensitivity of the collection of target DNA molecules when used in hybridisation-based assays.

When a collection of labelled target DNA molecules according to the invention is to be used in a hybridisation-based assay (e.g. to "probe" DNA molecules of reference samples) it may be preferred that the DNA molecules of the reference samples are also treated with exonuclease in order that they too may remain single-stranded. In this case it is important to note that the target DNA and reference DNA should be treated with exonucleases having complementary specificities. For example, in one embodiment the collection of labelled target DNA molecules may be produced using a 3'-5' exonuclease, and the reference samples treated with a 5'-3' exonuclease. In an alternative embodiment the collection of labelled target DNA molecules may be produced using a 5'-3' exonuclease, and the reference samples treated with a 3'-5' exonuclease.

Collections of labelled target DNA molecules according to the invention may be used in a range of detection techniques that are based upon the hybridisation of complementary nucleic acids. An example of a technique in which labelled target DNA molecules of the invention may be used is provided by our co-pending U.K. patent application (entitled "Analysis of Biological Samples") filed concurrently herewith, which is described below.

A collection of labelled target DNA molecules according to the invention may be used in a method of analysing a biological sample of interest, comprising:

(i) providing a collection of labelled target DNA molecules (acting as a "probe library") representative of a pattern of multiple gene expression in the biological sample of interest;

(ii) providing a plurality of individual reference samples each being a library comprised of cDNA or a derivative thereof representative of a pattern of gene expression in reference biological samples from which the reference samples have been derived;

(iii) treating individual reference samples with the collection of labelled target DNA molecules ("probe library") under hybridising conditions; and

(iv) determining the relative degree of hybridisation of the collection of labelled target DNA molecules ("probe library") to the reference samples, thereby providing an indication of the degree of similarity between gene expression in the biological sample of interest and gene expression in the individual reference biological samples.

The method of analysis described above makes it possible to obtain an indication of the degree of similarity of the pattern of gene expression in a sample of interest with that in a number of reference samples. The greater the degree of similarity of the pattern of gene expression in the sample of interest with a particular reference sample then the greater the similarity between these two samples. The ability to compare gene expression in a sample of interest with that in a large number of reference samples is, of course, particularly advantageous where the reference samples are of well characterised biological status since conclusions may then be drawn as to the biological status of the sample of interest.

A library for use according to the method of the invention is a collection of individual sequences representative of gene expression within the biological sample from which the library is derived. The number of sequences in the collection is sufficient to provide significant information about the biological activity or status of the biological sample from which the library is derived. Thus, although a biological sample may express many thousands of genes a library may, for instance, represent ten or more genes the expression

of which are characteristic of the activity or status of the biological sample. Preferably the probe library may represent twenty or more genes the expression of which are characteristic of the biological sample. Most preferably the probe library may represent fifty or more genes the expression of which are characteristic of the activity or status of biological sample.

More particularly the method of the invention utilises a probe library comprising cDNA, or a derivative thereof, representative of the mRNA content of, and hence a pattern of gene expression in, the sample of interest. The individual reference samples are each libraries comprised of cDNA molecules representative of gene expression in the biological samples from which the reference samples are derived. The libraries may, for example, comprise total cDNA. Examples of cDNA derivatives that may be employed in the method of the invention include sub-populations of total cDNA (e.g. obtained by complexity reduction techniques), and derivatives obtained by partial exonuclease digestion of the cDNA. The term cDNA derivative also includes RNA obtained from any form of DNA that may be employed in the invention.

The comparison of the patterns of gene expression in the sample of interest and the reference samples may be effected by probing the reference samples with the probe library under conditions allowing hybridisation of molecules in the two samples that are complementary to one another. It is preferred that the probe library is labelled for purposes of detecting hybridisation. If a particular gene is being expressed in both a sample of interest and a reference sample then the probe representing that gene will hybridise to the corresponding cDNA in the reference sample. This hybridisation may then be detected. The greater the level of hybridisation between the probe library and reference samples the greater the degree of similarity in the patterns of gene expression in the samples from which they are derived.

The ability to compare overall gene expression in a sample of interest with that in a number of reference samples is particularly advantageous when the reference samples are of defined and well-characterised biological status since conclusions may then be drawn as to the biological status of the sample of interest.

The method of the invention need only require a single round of hybridisation to allow comparison between the pattern of expression of a plurality of genes in a sample of interest with the pattern of expression of the same genes in a number of reference samples. The pattern of expression may potentially extend to the expression of thousands of different genes. Since known techniques only analyse the expression of either small numbers of genes or small numbers of samples such information could only be provided by the prior art on completing multiple rounds of hybridisation. Thus the method of the invention provides advantages both in terms of a reduction in the time necessary to perform such a comparison, and also in the reduced amount of reagents required.

In contrast to existing methodologies (in which specific probes are used to investigate the expression of specific genes) the method of the invention is able to compare patterns of gene expression without requiring any specific information as to the genes involved. Thus it is not necessary to identify those genes that may be of interest before comparing patterns of gene expression between unknown and reference samples. This provides a considerable advantage over the prior art in that an investigator does not need to know what gene (or genes) are involved in, for example, a particular response to a therapeutic agent before he can establish whether a test subject is likely to respond in a similar way to a previously characterised subject with a known response.

The reference samples may be derived from biological reference samples representing a number of different biological conditions or states. Alternatively the reference samples may be derived from biological reference samples representing a number of different examples of the same biological condition or state. Individual reference samples may be derived from biological samples taken from one or more individual. In the instance that a reference sample is derived from a single individual the reference sample may be derived from a biological sample representing a single tissue, or from biological samples representing a number of different tissues. In the case of reference samples derived from

biological samples taken from more than one individual the biological samples may all represent one type of tissue, or may represent a number of different tissue types. By including reference samples which share a common biological phenotype yet have arrived at that state via different routes the method of the invention is able to discriminate between treatment and biological status.

Examples of materials that may be used as reference samples include samples which are derived from patients with known clinical conditions and/or with known clinical outcomes.

In one such example reference samples may be taken from a number of patients with different forms of a particular disease. In this instance the sample of interest may be taken from an individual suspected of having, or being predisposed to, the disease in question. By comparing the pattern of gene expression in the sample of interest with the patterns of gene expression in the reference samples it is possible to establish which of the reference samples the sample of interest most closely resembles. This may then in turn provide an indication as to the particular form of the disease in question that the individual tested has or is predisposed to.

Alternatively the reference samples may be derived from patients with the same disease, but having different (known) reactions to different therapeutic agents. In this case comparison of the sample of interest and the reference samples will establish which of the patients with known treatment history the individual providing the sample of interest most resembles. This knowledge can then be used in order to select the treatment regime believed most likely to produce a beneficial outcome for the individual in question.

In another alternative reference samples may be derived from the same patient at different times, for instance before, during and after therapy. Comparison of such samples with a sample of interest taken from a patient with the same disease may be useful in assessing the progress of the patient of interest during treatment.

In a further alternative suitable reference samples may be collected from experimental subjects, such as animals or cultured cells, that have undergone procedures the effects of which are well studied.

For example reference samples may be collected from cells of a cell line that have been exposed to different drugs that have known effects (either on the cell line or on organisms of interest). These samples may then be probed using a probe library derived from cells of the same cell line that have been exposed to a putative drug which has an unknown effect. By comparing gene expression patterns established in response to the known and unknown drugs it is then possible to establish which of the known drugs the unknown drug most resembles. This will provide an indication that the effects of the unknown drug are likely to be similar to those of the known drug that it most closely resembles.

In a further example the sample of interest may be taken from tissues of experimental animals that have undergone treatments bringing about conditions that resemble those of a disease of interest. The pattern of gene expression in these samples may then be compared with the pattern of gene expression in reference samples taken from normal biological samples or biological samples from individuals suffering from the disease in question in order to investigate how changed gene expression influences the particular disease. Suitable experimental animals may, for instance, include transgenic animals, such as animals in which certain genes have been up-regulated, down-regulated or deleted.

In another application of the method of the invention the sample of interest may be taken from a tissue that includes, or may be thought to include, a cell type of particular interest. Such cells may, for example, be stem or progenitor cells. In this case tissues representing suitable biological samples from which reference samples may be derived will include tissues known to contain the cell type of interest, or tissues known to contain specific forms of the cell type of interest. Comparison of the pattern of gene expression in the sample of interest with the pattern of gene expression in the reference samples may indicate that the sample of interest either does or does not contain the cell type of interest. If the sample of interest contains cells of the cell type of interest then the method of the

invention may also provide information as to the number, form or status of these cells present in the sample.

In the field of stem cell biology defining the specific gene expression changes in stem cells, their immediate daughter cells, cells committed to differentiation and fully differentiated cells under conditions that alter self-renewal and differentiation represents a powerful means of identifying potential drug targets. For example, the discovery of a growth factor or growth factor receptor specifically expressed in stem cells undergoing increased self-renewal would lead to the development of pharmacological approaches designed to inhibit stem cell expansion during cancer development or increase stem cell expansion following injury. Furthermore, identification of genes whose expression is specifically linked to eventual stem cell self-renewal and differentiation will greatly facilitate the monitoring of stem cell behaviour that is an essential component of pre-clinical drug evaluation.

Since the method of the invention compares the patterns of expression of a number of genes within test and reference samples it is particularly well suited to the study and comparison of biological activities associated with stem cells that involve the interplay of a number of different genes, for example in a biochemical pathway. Using known methods to investigate such interactions it would be necessary to identify each gene involved in a pathway and conduct separate hybridisation reactions to determine the expression of each gene. The method of the invention, in contrast will in a single round of hybridisation report on the comparative expression of all genes involved, even including genes that may not be known to be associated with the pathway.

Conveniently the method of the invention may be effected using an array or microarray on a solid substrate. Either the probe library or the reference samples may be provided as an array or microarray on a suitable substrate. Preferably it is the reference samples that are provided as the array on the substrate. In a particularly preferred embodiment an array or microarray may comprise a library of reference samples containing DNA samples derived from groups representative of different biological conditions, each group containing samples derived from a number of different individuals sharing the same

condition, wherein the DNA samples are arranged in order on the array or microarray such that members of the same group are located in proximity to one another. The DNA samples may, for instance, be arranged in order in a grid pattern such that each row of the grid represents a group of individuals sharing the same biological condition.

A suitable microarray may, for instance, be produced on a substrate such as glass, a silica-based chip, a nylon membrane or a microtiter plate. Many examples of techniques suitable for the manufacture of arrays or microarrays will be readily apparent to those skilled in the art. These include the techniques disclosed in Maniatis et al. 1982, Chee et al. 1996, Iyer et al. 1999, Lipshutz et al. 1995, Lockhart et al. 1995, Schena 1996, Schena et al. 1995, Soares et al. 1997 and Southern 1996.

DNA may be coupled to the support material forming the array by means of electrostatic interaction with a coating film of a polycationic polymer such as poly-L-lysine (as described in WO 95/35505) or may be covalently bound to the support by well established techniques.

In an alternative embodiment the method of the invention may, if preferred, be performed with both the probe library and reference samples in free solution.

Biological samples suitable for use according to the invention include any sample containing material representative of gene expression in the sample, such as mRNA. Biological samples preferably comprise biological cells, indeed a suitable biological sample may even comprise a single cell. Suitable samples may be taken by means of biopsies, swabs, hair or skin samples, or as samples of bodily fluids such as blood, cerebrospinal fluid (CSF), saliva, milk, faeces and urine. In particular samples for use in analysis of stem cells may suitably be taken from foetal or embryonic tissue, from bone marrow or from germ cells or from other tissues in the adult or developing organism.

The probe library and the reference samples may preferably comprise, or be derived from, global amplified cDNA.

Materials that may be derived from total cDNA include sub-populations of the total cDNA, truncated or otherwise manipulated versions of the cDNA and other materials representative of patterns of gene expression produced using the total cDNA as a template.

The invention may be effected using probe libraries and/or reference samples comprising cDNA produced as described above without further modification. However, various modifications may be made what will improve the sensitivity of the method.

Complexity reduction techniques may also be used in preparation of the probe library and/or reference samples to improve the sensitivity of the method of the invention. The rationale behind such techniques is that many of the mRNAs present in a biological sample, such as the sample of interest or the reference samples, represent transcription of so-called "house keeping" genes. These genes encode products associated with the up-keep of the cell and are generally likely to be common to both samples of interest and reference samples. As such they represent components of gene expression patterns that may be found in both test and reference samples, but which are unlikely to be important in the development or maintenance of a biological condition or state of interest. It has been estimated that up to 65% of mRNA mass within cells may be composed of transcripts representing "house-keeping" genes.

Complexity reduction techniques improve sensitivity either by simply reducing the number of individual genes represented in the probe library and/or reference samples, or by specifically removing irrelevant or "house keeping" genes from the probe library and/or reference samples. Thus the relative abundance of those molecules representative of gene expression that remain after application of a complexity reduction technique is increased, thereby increasing the "signal to noise" ratio.

A number of complexity reduction techniques may be used in effecting the method of the invention. These techniques may be used in isolation or in combination. Preferably the same complexity reduction technique, or combination of complexity reduction techniques, are used to treat the cDNA, or its derivatives, to produce both the probe

library and the reference samples, although it is possible to apply complexity reduction techniques to only one of the DNA populations.

Suitable examples of complexity reduction techniques include:

Restriction enzyme based.

In this complexity reduction technique site specific endonucleases are used to digest the cDNA or its derivatives. Since the frequency of cleavage sites for any specific endonuclease will depend on the size and base composition of the cleavage site endonucleases can be chosen that will cut a sub-set of all DNA molecules present. For example, a restriction endonuclease recognising a six base site will, on average, cleave every 4,096 base pairs. Thus in a DNA population in which the average polynucleotide size is 2,000 bases such a endonuclease will cleave approximately half of all polynucleotides present. Following restriction digestion either the cleaved products or the uncleaved products can be selectively enriched. By choosing the appropriate restriction enzymes distinct subsets of cDNA sequences can be either eliminated or enriched. By applying this type of strategy the initial total cDNA sample can divided into subsets of genes whereby each sequence is effectively enriched making it more likely that changes in each individual gene will be detected during array hybridisation.

Thus for individual gene sequence present after applying complexity reduction there will be an increase in specific activity for each gene and an increase in the "signal to noise".

Display products.

Another means of selecting a subset of sequences present in the starting cDNA/mRNA population, and thereby increasing the relative abundance of each selected sequence after complexity reduction, is the use of approaches for differential cDNA display (Liang and Pardee, 1992). cDNA display selectively amplifies only those cDNA populations which contain effective priming sites for display primer(s) used. Display primers can be used to prepare distinct subsets of cDNAs directly from starting RNA (Liang and Pardee, 1992) or alternatively display amplification may be applied to amplified total cDNA populations (Candeliere et al., 1999). In essence display techniques reduce complexity by

selectively enriching a subset of the sequences present in the original mRNA or cDNA population, thereby increasing the relative abundance of the selected sequences within the resultant population.

Hybridisation depletion and enrichment.

A variety of DNA/RNA subtraction techniques have been developed to deplete DNA/RNA sequences common to two or more pools of DNA/RNA molecules. DNA/RNA subtraction applied to DNA or RNA copies (either direct copies or amplified products) of the original extracted RNA can be used to reduce complexity by removing sequences.

Suitable DNA/RNA subtraction techniques for use according to the invention are well known. One such method involves the production of a single-stranded cDNA library (the "tracer"), such as the cDNA from which the probe library or reference samples are to be generated, from which it is desired to remove certain sequences. A collection of amplified cDNAs representing the sequences that one wishes to eliminate (the "driver"), such as housekeeping genes, is then allowed to hybridise with the tracer. Double stranded DNA molecules, representing hybrids of the tracer and the driver, may then be removed from the total population of DNA based upon their adhesion to hydroxyapatite. The remaining DNA population comprises single stranded DNA molecules representing the tracer population minus the driver population. This subtracted DNA population may then be further amplified as required.

In further refinements of this method "driver" nucleic acids may be covalently linked to compounds which facilitate the physical separation of "driver" nucleic acids (plus any annealed "tracer") from unhybridised "tracer". The separated populations (i.e. those sequences present only in the "tracer", or those sequences shared by both "tracer" and "driver") may then be enriched or depleted relative to one another. For example, driver nucleic acids may be linked to biotin, such that following hybridization all biotinylated hybrids can by segregated by interaction with immobilised avidin, allowing either subtractive enrichment or positive selection. Suitable protocols are described in Welcher

et al., 1986; and Weaver et al., 1999. In alternative, but similar, approaches "driver" nucleic acids may be bound to latex beads (as described in Kuribayashi-Ohta et al., 1993, or magnetic particles (as described in Lopez-Fernandez and del Mazo, 1993; and Schraml et al. 1993.

In one embodiment hybridisation depletion/enrichment protocols can be used to remove "unwanted sequences" present in samples from which the probe library and/or reference samples are derived. The nature of the "unwanted sequences" will depend on the biological samples in question. However, as a general rule, sequences which are expressed at similar levels in diverse samples are, by their very nature, uninformative and tend simply to add to the "background" produced during hybridisation.

It is likely that genes expressed at a similar level in biologically divergent tissues will not be characteristic of a particular tissue, and will instead represent house-keeping genes. By way of example, it is unlikely that genes expressed at a similar level in tissues as biologically different as heart, lung, spleen and testes will be characteristic of any one of these tissues. Sequential hybridisation enrichment can be used to obtain a "pool" of sequences common to different tissues. The resultant pool will represent genes that contribute to the "background noise" associated with hybridisation. This pool can then be expanded and used to reduce the level of background hybridisation. For example, it is possible to subtract these common sequences from both the probe library and reference samples, thereby reducing the level of total hybridisation. Alternatively it is possible to use the pool of common genes to produce unlabelled competitor DNA and thereby reduce the level of detectable hybridisation.

Using probe libraries and reference samples produced in accordance with the techniques described above the method of the invention may be effected by reference samples and probe library under hybridising conditions. The conditions under which nucleic acids will hybridise to one another are well known to those skilled in the art. Specific conditions are described in greater detail in the accompanying Example. Further examples of conditions suitable for nucleic acid hybridisation can be found in reference works such as "Molecular Cloning: A Laboratory Manual" edited by Maniatis et al.. Other suitable conditions are

described in Chee et al. 1996, Iyer et al. 1999, Lipshutz et al. 1995, Lockhart et al. 1995, Schena 1996, Schena et al. 1995, Soares et al. 1997 and Southern 1996.

Similarly, methods for determining the relative degree of hybridisation between populations of nucleic acids are also well known. Methods suitable for effecting the invention include labelling of the probe library with reporters such as fluorescent labels, radioactive labels or chromogenic enzymes. If the reference sample libraries are unlabelled then detection of the chosen label (after removing unbound probe) will confirm the presence of hybridisation between the sample of interest and the reference sample. Suitable techniques for labelling of the molecules comprising the probe library, for detection of hybridised probe and reference DNA molecules and for interpretation of hybridisation data are well known to those skilled in the art. These techniques include those described in Maniatis et al. 1982, Chee et al. 1996, Iyer et al. 1999, Lipshutz et al. 1995, Lockhart et al. 1995, Schena 1996, Schena et al. 1995, Soares et al. 1997 and Southern 1996.

Use of unlabelled competitor DNA.

When the probe library DNA is labelled and the reference sample DNA is unlabelled the sensitivity of the method of the invention may be improved by the use of unlabelled "competitor" DNA which can compete with the DNA of the probe library for hybridisation with the reference samples. The competitor DNA may be DNA representing common housekeeping genes, or it may be selected DNA representing genes common to the biological sample of interest and/or the reference samples. Since the competitor DNA is unlabelled, hybrids of competitor and reference DNA will not be detected in assessing total hybridisation.

The competitor DNA may be exposed to the reference sample DNA before the addition of the probe library DNA or at the same time as the addition of the probe library DNA. Molecules of the competitor DNA that represent genes expressed by the reference samples will then hybridise to the corresponding DNA of the reference samples. Reference sample molecules that undergo hybridisation with molecules of the competitor DNA will therefore be unable to hybridise with further molecules from the probe library.

Thus by incubating the DNA of the reference samples with, for example, unlabelled competitor DNA representative of housekeeping genes it is possible to reduce the level of binding by labelled probe DNA representing the same genes. This therefore improves the sensitivity of the method of the invention since it increases the likelihood that detected hybridisation is representative of genes of interest within the samples.

Unlabelled competitor DNA representative of genes having a high frequency of expression within the biological sample of interest and/or reference samples may be generated by reverse subtraction of the DNA populations derived from the two samples.

The present invention will now be illustrated by way of example only with reference to the accompanying drawing, illustrating use of the collection of labelled target DNA molecules as a "probe library" in the method described above, in which:

Figure 1a represents a schematic depiction of an array of reference samples suitable for use in the method of the invention before effecting hybridisation;

Figure 1b represents the same array after effecting hybridisation of a probe library with the reference samples;

Figure 1c represents a flow chart indicating suitable methods for producing a probe library and reference samples according to the invention; and

Figure 1a shows an array (1) provided with individual reference samples (2) derived from cDNA generated from biological reference samples. Each individual reference sample is a library representative of a pattern of gene expression in the biological reference sample. The rows of reference samples (2) on the array (1) each represent a distinct biological condition or state. Each reference sample (2) within a row is derived from a different individual sharing the same biological condition or state.

Figure 1b shows the results of probing the reference samples (2) on the array (1) with a labelled probe library according to the method of the invention. Sequences present within

both the probe library and the reference samples (2) hybridise to one another. Hybridisation is measured by colour development, hence the greater the degree of hybridisation between the probe library and a reference sample the more intense the colour. Thus in Figure 2a it can be seen that the probe library exhibits the greatest degree of similarity (and so hybridisation) with the reference samples of row 10, a lesser degree of similarity (and hybridisation) with reference samples of rows 3 and 6 and a still lesser degree of similarity with the reference samples of rows 1 and 8. The probe library does not share any sequences in common with the other rows of reference samples (2) and thus does not hybridise with these reference samples, so producing no colour development.

The probe library and reference samples may be prepared by the procedures illustrated in Figure 1c, in which RNA (3) from a biological sample of interest is amplified according to known protocols to generate global cDNA (4). This global cDNA (4) may then be used directly to produce a probe library (as indicated by arrow 5) or, more preferably, is subjected to complexity reduction techniques (6) prior to probe library production.

Complexity reduction (6) may, for instance, take the form of processing to display products (7), subtraction of unwanted sequences from the global cDNA generated from the sample of interest (8) or restriction digest of sequences in the cDNA generated from the sample of interest (9). The cDNA generated from the sample of interest may be subject to a combination of complexity reduction techniques (e.g. subtraction (8) and restriction digest (9)) or may be used to produce a probe library after a single complexity reduction technique.

Optionally, the cDNA, or derivative, of the probe library may be subject to exonuclease digestion in order to improve the sensitivity of the invention. This digestion may be effected either before or after complexity reduction.

Production of the probe library is completed by using known techniques to label (10) the cDNA generated from the sample of interest.

Although the generation and processing of cDNA has been described above with reference to production of the probe library, the techniques described (with the exception of labelling the probe library) are all equally suitable for production of reference samples from biological reference samples in order to produce a suitable array (11). Preferably both the probe library and reference samples to be used according to the method of the invention are produced using the same complexity reduction techniques. In the situation that both the probe library and reference samples are to be subject to exonuclease digestion the two different cDNA populations should be treated with exonucleases having different specificities, i.e. one treated with a 5' to 3' exonuclease, and the other treated with a 3' to 5' exonuclease.

Protocols.

The following Protocols are suitable for effecting the method described above using a collection of labelled target DNA molecules according to the invention as a "probe library".

(a) Preparation of global amplified cDNA
  (i) Preparation of cDNA
  (ii) Terminal transferase – "Tailing"
  (iii) Global cDNA amplification
(b) Preparation of array of reference samples
(c) Labelling of probe library
  (i) Terminal Transferase labelling
  (ii) PCR labelling
(d) Exonuclease treatment of double-stranded DNA
(e) Hybridisation of probe library and reference samples
(f) Detection of hybridisation
(g) Complexity reduction.
  (i) Display Based
  (ii) Hybridisation depletion and enrichment

(a) Preparation of global amplified cDNA.

The protocol described below is based on protocols described in Brady et al. (1990) and Brady, G., and Iscove, N. N. (1993).

Suitable starting materials include total RNAs, which may be prepared from biological tissues of interest (using commercially available kits such as those manufactured by Clontech), or mRNA present in biological cells ("direct amplification").

(i) Preparation of cDNA.

cDNA may be prepared from the mRNA from the biological tissues according to the following protocol:

1. RNAs are adjusted to 100 microgram/ml in 10 mM Tris pH 7.5, 1 mM EDTA

2. 3 µl of each RNA is added to 3 µl of the following buffer:

100 mM	Tris pH 8.3
150 mM	KCl
6 mM	$MgCl_2$
0.2 mg/ml	Glycogen (Roche)
2 %	NP-40 (Roche)
2.5 nM	dNTPs (Sigma)
0.75 µM	dT24 (Sigma/Genosys)
0.37 u/ml	RNAse inhibitors (Ambion)

3. Samples are heated to 65°C for 1 minute allowed to cool at RT for 3 minutes then placed on wet ice

4. After 1 to 10 minutes on ice 3 µl of the following buffer containing 85 u MMLV RTase and 1 u AMV RTase is added to each sample:

50 mM	Tris pH 8.3
75 mM	KCl
3 mM	$MgCl_2$
0.1 mg/ml	Glycogen (Roche)
1 %	NP-40 (Roche)

5. Samples are Incubated 15 minutes at 37°C, heat inactivated at 65°C for 10 minutes then cooled to 4°C.

(ii) Terminal Transferase – 'Tailing'

1. 5 µl of each sample is mixed with 5 µl of the following buffer containing 2.3 units terminal transferase.

200 mM	potassium cacodylate pH 7.2
4 mM	$CoCl_2$
0.4 mM	DTT
1 mM	dATP

2. Samples are then incubated 15 minutes at 37oC, 65oC 10 minutes and cooled to 4oC.

(iii) Global cDNA amplification.

Global cDNA prepared from biological tissues according to the preceding protocols may be amplified according to the following protocol:

1. 8 µl of the tailed cDNA prepared as described above may be combined with 8 µl of:

121.4 mM	KCl
8.5 mM	$MgCl_2$
24.25 mM	Tris-HCl pH 8.3

48 µg/ml	Glycogen (Roche)
2.4 %	Triton X-100
2.3 mM	dNTPs
9.6 µM	Oligo Not1dT (sequence CATCTCGAGCGGCCGCTTTTTTTTTTTTTTTTTTTTTT)
0.16 u/µl	Taq Polymerase

2. Samples are then placed into a PCR machine and subjected to:

25 cycles
1 minute 94°C
2 minute 42°C
6 minute 72°C

followed by an additional 25 cycles:
1 minute 94°C
1 minute 42°C
2 minute 72°C

3. Following completion of PCR samples are purified using the Millipore 96 well purification system (Millipore MANU 03050) following instructions provided by the manufacturer.

(b) Preparation of array of reference samples.

An array comprising global purified cDNA (prepared as described above) may be produced using the following protocol:

Purified global cDNAs from heart, lung, spleen and testes may separately be adjusted to around 50 ng/µl in 50% DMSO, boiled and spotted in groups of 12 onto CMTGAPS glass slides (Corning) using a Gene Machines OmniGrid as recommended by the manufacturer.

(c) Labelling of probe library.

The following provides suitable protocols for labelling of probe library cDNA for use according to the method of the invention. The following protocols describes the labelling of two different cDNA populations (which may be prepared using the protocols described above) with two different fluorescent markers (Cy3 and Cy5).

(i) Terminal Transferase labelling.

1. Approximately 50 ng of globally amplified cDNA of a first probe library may be added to a 20 µl reaction containing:

100 nM	FluoroLink™ Cy3-dUTP (Amersham Pharmacia Biotech)
100 mM	potassium cacodylate pH 7.2
2 mM	$CoCl_2$
0.2 mM	DTT

total 5 units	Terminal Transferase

2. Approximately 50 ng of globally amplified cDNA of a second probe library may be added to a 20 µl reaction containing:

100 nM	FluoroLink™ Cy5-dUTP (Amersham Pharmacia Biotech)
100 mM	potassium cacodylate pH 7.2
2 mM	$CoCl_2$
0.2 mM	DTT
total 5 units	Terminal Transferase

3. Following incubation for 1 hour at 37°C both samples may be ethanol precipitated by the addition of:

10 µl	7.5 M Ammonium Acetate
0.5 µl	15 mg/ml Glyco Blue (Ambion)
75 µl	ethanol

Samples may then be held on wet ice for 15minutes, centrifuged at 4°C at 14,000 rpm for 20 minutes and the pellets washed twice with 70% ethanol, allowed to dry 10 minutes at room temperature then resuspended in 5 µl 10 mM Hepes pH 7.5, 1 mM EDTA.

(ii) PCR labelling.

Further rounds of PCR amplification can be used to incorporate fluorescent markers directly or indirectly coupled to nucleotides present in the PCR reaction. An example of such an approach is given below.

1. Approximately 0.5 ng of globally amplified cDNA of a first probe library may be added to a 20-100 µl reaction containing:

100 nM	FluoroLink™ Cy3-dUTP (Amersham Pharmacia Biotech)
100nM	dNTPs
1 µM	Oligo NotIdT (sequence CATCTCGAGCGGCCGCTTTTTTTTTTTTTTTTTTTTTT)
16mM	$(NH_4)_2SO_4$
67mM	Tris-HCl (pH 8.8 at 25°C)
0.01%	Tween-20
0.16 u/µl	Taq Polymerase

2. Approximately 0.5 ng of globally amplified cDNA of a second probe library may be added to a 20-100 µl reaction containing:

100 nM	FluoroLink™ Cy5-dUTP (Amersham Pharmacia Biotech)
100nM	dNTPs

1 µM	Oligo Not1dT (sequence CATCTCGAGCGGCCGCTTTTTTTTTTTTTTTTTTTTTTT)
16mM	$(NH_4)_2SO_4$
67mM	Tris-HCl (pH 8.8 at 25°C)
1.5 mM	$MgCl_2$
0.01%	Tween-20
0.16 u/µl	Taq Polymerase

3. Both samples are then placed into a PCR machine and subjected to:

25 cycles	
30 seconds	94°C
1 minute	42°C
2 minutes	72°C

4. Following both samples may be ethanol precipitated by the addition of:

0.5 original sample volume	7.5 M Ammonium Acetate
0.025 original sample volume	15 mg/ml Glyco Blue (Ambion)
3.5 original sample volumes	ethanol

Samples may then be held on wet ice for 15minutes, centrifuged at 4°C at 14,000 rpm for 20 minutes and the pellets washed twice with 70% ethanol, allowed to dry 10 minutes at room temperature then resuspended in 5 µl 10 mM Hepes pH 7.5, 1 mM EDTA.

(d) Exonuclease treatment of double-stranded DNA.

Note exonuclease treatment can be applied to either freshly amplified cDNA or labelled cDNA.

(i) 3'-5'-exonuclease - Exonuclease III

1. Add freshly 0.5 – 5 µg cDNA to a 50 µl reaction consisting of:

660mM	Tris pH 8.0
6.6mM	$MgCl_2$
5 mM	DTT
50 µg/ml	BSA
10 units	Exonuclease III (Amersham Pharmacia)

2. Incubate 30 minutes at 37°C.

3. Following heat inactivation at 75°C for 30 minutes ethanol precipitate by the addition of:

0.5 original sample volume	7.5 M Ammonium Acetate
0.025 original sample volume	15 mg/ml Glyco Blue (Ambion)
3.5 original sample volumes	ethanol

Samples may then be held on wet ice for 15minutes, centrifuged at 4°C at 14,000 rpm for 20 minutes and the pellets washed twice with 70% ethanol, allowed to dry 10 minutes at room temperature then resuspended in 5 µl 10 mM Hepes pH 7.5, 1 mM EDTA.

(ii) 5'-3'-exonuclease - T7 Gene 6 Exonuclease

1. Add freshly 0.5 – 5 µg cDNA to a 50 µl reaction consisting of:

40 mM	Tris pH7.5
20 mM	$MgCl_2$
50 mM	NaCl
10 units	T7 Gene 6 Exonuclease (Amersham Pharmacia)

3. Incubate 30 minutes at 37°C.

3. Following heat inactivation at 75°C for 30 minutes ethanol precipitate by the addition of:

0.5 original sample volume	7.5 M Ammonium Acetate
0.025 original sample volume	15 mg/ml Glyco Blue (Ambion)
3.5 original sample volumes	ethanol

Samples may then be held on wet ice for 15minutes, centrifuged at 4°C at 14,000 rpm for 20 minutes and the pellets washed twice with 70% ethanol, allowed to dry 10 minutes at room temperature then resuspended in 5 µl 10 mM Hepes pH 7.5, 1 mM EDTA.

(e) Hybridisation of probe library and reference samples.

Hybridisation of probe library and reference samples according to the method of the invention may be effected as follows, using an array and probe libraries prepared as described above.

1. An array slide may be prehybridised at 42°C for 1 hour in the following buffer:

50% Formamide
5X SSC
0.1 % SDS
10 mg/ml BSA

2. The array slide may then be washed four times with H_2O and once in Isopropanol and dried 5 minutes at room temperature.

3. The following mixture may then be prepared:

50%v/v	Formamide
5X	SSC

0.1 % SDS
0.5mg/ml Poly A RNA
0.5mg/ml Yeast tRNA
0.5mg/ml Salmon Sperm DNA (10-30ug)
50ug/ml Cot1 DNA
combined Cy3 and Cy 5 probes

(Total volume 45 µl)

4. The mixture may then be heated at 95°C for 5 minutes and chilled on wet ice 3 minutes.

5. The mixture may be applied to a cover slip and the pre-warmed (42°C) array slide (arrayed material facing downwards) lowered onto cover slip to the point when it is just possible to lift the cover slip up with surface tension.

6. The slide may be placed into a moisturised slide hybridisation chamber and incubated 42 °C o/n.(<16hr).

7. Following hybridisation the entire slide may be immersed in 2X SSC and the cover slip removed.

8. The exposed slide may then be washed twice 2X SSC/0.1% SDS (5 minutes RT each wash) followed by 2 washes with 2X SSC (5 minutes RT each wash) and drying at room temperature.

(f) Detection of hybridisation.

The following protocol is suitable for detection and analysis of hybridisation in the method of the invention.

1. Scanning of the slide and quantification of red (Cy5 635nm) and green (Cy3 532nm) fluorescence may be carried out using a GenePix 4000b as recommended by the manufacturer.

2. Following scanning data may be analysed using commercially available software.

(g) Complexity reduction.

There are many possible complexity reduction techniques that are suitable for use with the method of the invention.

(i) Display based

The following protocol is suitable for effecting a "display products" complexity reduction technique according to the method of the invention. The protocol provides for the preparation of two different amplified cDNA populations from the same cDNA population ("total cDNA").

Selected subsets of cDNA within a global amplified total cDNA population may be further amplified based on protocols described in:

Candeliere, G. A., Rao, Y., Floh, A., Sandler, S. D., and Aubin, J. E. (1999). cDNA fingerprinting of osteoprogenitor cells to isolate differentiation stage-specific genes. Nucleic Acids Research *27*, 1079-83.

A suitable protocol is as follows:

1. Purified globally amplified total cDNA prepared as described above may be diluted 100 fold in 2 mM Tris pH 7.5, 0.2 mM EDTA.

2. Two separate subsets of cDNAs may then be selectively amplified from the total cDNA by separately adding 10 µl of total cDNA to 10 µl of PCR mixture A and 10 µl of total cDNA to 10 µl of PCR mixture B, and subjecting both to:

2 cycles as follows:
94°C 1 minutes;
35°C 3 minutes;
72°C 3 minutes;

followed by 30 cycles as follows:

94°C 30 seconds;

50°C 30 seconds;

72°C 1 minute; and

1 cycle as follows:

72°C 5 minutes.

PCR mixture A

25 µM	Display Oligo A – CAGCCAGTCTTGAGGCAACACC
0.5 mM	dNTPs (Sigma)
32 mM	$(NH_4)_2SO_4$
134 mM	Tris-HCl (pH 8.8 at 25°C)
0.01%	Tween-20
3 mM	$MgCl_2$
25 u/ml	Taq Polymerase

PCR mixture B

25 µM	Display Oligo B – CCAGCAAGAGCACAAGAGGAAGAG
0.5 mM	dNTPs (Sigma)
32 mM	$(NH_4)_2SO_4$
134 mM	Tris-HCl (pH 8.8 at 25°C)
0.01%	Tween-20
3 mM	$MgCl_2$
25 u/ml	Taq Polymerase

Following PCR all samples may be purified using GFX purification columns (Amersham Pharmacia) following the manufacturer's instructions.

(ii) Hybridisation depletion and enrichment

The term *driver* refers to the cDNA used to deplete and/or enrich in the *tracer* cDNA population. The resultant depleted or enriched sequences will be derived from the *tracer* cDNA population. In the following examples all *driver* cDNAs are prepared in PCR reactions containing dUTP (not dTTP) to allow removal of residual *driver* cDNAs using the dUTP specific UNG nuclease.

Based on methods described in:
Analysis of gene-expression in a complex differentiation hierarchy by global amplification of cdna from single cells. Brady, G, Billia F, Knox J, Hoang T, Kirsch IR, Voura EB, Hawley RG, Cumming R, Buchwald M, Siminovitch K, Miyamoto N, Boehmelt G, and Iscove NN: *Current Biology* 1995, 5: 909-922.

Foot, HCC, Brady G, and Franklin FCH. (1996). Subtractive Hybridisation. In Plant Molecular Biology Laboratory Manual, M. Clark, ed. (London: Springer Verlag).

Weaver, DL, Núñez C, Brunet C, Bostock V, and Brady G. (1999). Single-cell RT-PCR cDNA subtraction. In Molecular Embryology: Methods and Protocols., P. Sharpe and I. Mason, eds. (Totowa, NJ, USA: Humana Press), pp. 601-609.

Depletion/Subtraction

1. Preparation of tracer and driver:

Tracer

Approximately 0.5 ng of globally amplified cDNA added to a 20-100 µl reaction containing:

250 nM	dATP, dTTP, dCTP, dGTP
1 µM	Oligo NotIdT (sequence CATCTCGAGCGGCCGCTTTTTTTTTTTTTTTTTTTTTT)
16mM	$(NH_4)_2SO_4$
67mM	Tris-HCl (pH 8.8 at 25°C)
1.5 mM	$MgCl_2$
0.01%	Tween-20
0.16 u/µl	Taq Polymerase

Driver

Approximately 0.5 ng of globally amplified cDNA added to a 20-100 µl reaction containing:

250 nM	dATP, dUTP, dCTP, dGTP
1 μM	Oligo Not1dT (sequence CATCTCGAGCGGCCGCTTTTTTTTTTTTTTTTTTTTTT)
16mM	$(NH_4)_2SO_4$
67mM	Tris-HCl (pH 8.8 at 25°C)
1.5 mM	$MgCl_2$
0.01%	Tween-20
0.16 u/μl	Taq Polymerase

Both *tracer* and *driver* are then placed into a PCR machine and subjected to:

25 cycles	
30 seconds	94°C
1 minute	42°C
2 minutes	72°C

Following completion of the PCR reaction both *tracer* and *driver* cDNAs are then purified using commercial purification systems such as GFX (Amersham Pharmacia).

Biotinylation of Driver.

Place 20-50 μl driver DNA (2-50 μg) in a 1.5 ml screw-cap tube. Boil for 2 minutes and place directly on ice in a small ice tray + rack.

Add 20 μl 2 mg/ml photobiotin to the DNA and mix well. With the lids left off place the tubes upright on ice 10 cm from the bulb and irradiate for a total 10 minutes. After the first 5 minutes remove the tubes from under the light source (avoid direct irradiation), flick the tube to mix and replace under the light source for the remaining 5 minutes.

Remove the sample (avoid direct irradiation) and mix in the remaining 20 μl of photobiotin and place under the light for an additional 5 minutes.

Add 1/10th volume of 1M Tris-Cl, pH 8.0 to stop the reaction.

Purify using commercial purification systems such as GFX (Amersham Pharmacia).

2. Hybridisation of *tracer* plus *driver* and *tracer* enrichment:

To a 0.5 ml tube add and mix in this order:

0.5 µg	*tracer DNA*
10 µg	biotinylated *driver DNA*

adjust volume to 20 µl with water then add:

8 µl	5xHyb *GEH*
12 µl	40 % PEG

Heat sample:

5 minutes 98°C,
5 minutes at 80°C
7 minutes at 74°C
60 minutes at 68°C
then hold at 68°C while seperating biotinylated molecules

Remove biotinylated molecules using avidin bound to a solid support. In practise this can be carried out using commercial products as ditrected by the manufacturer such as Streptavidin Magnasphere™ Paramagnetic particles (SA-PMPs) provided by Promega.

Following removal of biotinylated molecules the remaining *tracer* can be subjected to further rounds of subtraction by addition of fresh biotinylated *driver DNA* and repeating the process described above. Typically three sequential rounds of subtraction are used but additional rounds may be added if required.

The final depleted product is then amplified using PCR conditions described for the original tracer amplification.

5xHyb *GEH*

90 mM	EPPS pH 8.5

10 mM	EDTA pH 8.0
0.5 %	Triton X-100
3.75 M	NaCl

Negative Subtraction or Attraction

1. Preparation of tracer and driver:

Tracer

Approximately 0.5 ng of globally amplified cDNA added to a 20-100 µl reaction containing:

250 nM	dATP, dTTP, dCTP, dGTP
1 µM	Oligo Not1dT (sequence CATCTCGAGCGGCCGCTTTTTTTTTTTTTTTTTTTTTT)
16mM	$(NH_4)_2SO_4$
67mM	Tris-HCl (pH 8.8 at 25°C)
1.5 mM	$MgCl_2$
0.01%	Tween-20
0.16 u/µl	Taq Polymerase

Driver

Approximately 0.5 ng of globally amplified cDNA added to a 20-100 µl reaction containing:

250 nM	dATP, dUTP, dCTP, dGTP
1 µM	Oligo Not1dT (sequence CATCTCGAGCGGCCGCTTTTTTTTTTTTTTTTTTTTTT)
16mM	$(NH_4)_2SO_4$
67mM	Tris-HCl (pH 8.8 at 25°C)
1.5 mM	$MgCl_2$
0.01%	Tween-20
0.16 u/µl	Taq Polymerase

Both *tracer* and *driver* are then placed into a PCR machine and subjected to:

25 cycles	
30 seconds	94°C
1 minute	42°C
2 minutes	72°C

Following completion of the PCR reaction both *tracer* and *driver* cDNAs are then purified using commercial purification systems such as GFX (Amersham Pharmacia).

Biotinylation of Driver

Place 20-50 µl driver DNA (2-50 µg) in a 1.5 ml screw-cap tube. Boil for 2 minutes and place directly on ice in a small ice tray + rack.

Add 20 µl 2 mg/ml photobiotin to the DNA and mix well. With the lids left off place the tubes upright on ice 10 cm from the bulb and irradiate for a total 10 minutes. After the first 5 minutes remove the tubes from under the light source (avoid direct irradiation), flick the tube to mix and replace under the light source for the remaining 5 minutes.

Remove the sample (avoid direct irradiation) and mix in the remaining 20 µl of photobiotin and place under the light for an additional 5 minutes.

Add 1/10th volume of 1M Tris-Cl, pH 8.0 to stop the reaction.

Purify using commercial purification systems such as GFX (Amersham Pharmacia).

2. Hybridisation of *tracer* plus *driver* and *tracer* enrichment:

To a 0.5 ml tube add and mix in this order:

0.5-10 µg *tracer DNA*

10 µg biotinylated *driver DNA 1*

adjust volume to 20 µl with water then add:

8 µl 5xHyb *GEH*

12 µl 40 % PEG

Heat sample:

5 minutes 98°C,
5 minutes at 80°C
7 minutes at 74°C
60 minutes at 68°C
then hold at 68°C while seperating biotinylated molecules

Remove biotinylated molecules using avidin bound to a solid support. In practise this can be carried out using commercial products as ditrected by the manufacturer such as Streptavidin Magnasphere™ Paramagnetic particles (SA-PMPs) provided by Promega.

Release *tracer DNA* bound to *driver DNA 1* by denaturing the *driver DNA 1/tracer DNA* hybrids. For examples using SA-PMPs the washed SA-PMPs and their attendant *driver DNA1/tracer DNA* hybrids can be heated to 96°C to release *tracer DNA* and bound *driver DNA 1* removed by magnetic attraction of the SA-PMPs.

Released *tracer DNA* can then be subjected to further rounds of attraction by repeating the process with separate drivers (*driver DNAs 2, 3, 4* etc).

The final "attracted" product will be enriched for sequences common to all *driver DNAs* used and can be amplified using PCR conditions described for the original tracer amplification.

5xHyb *GEH*

90 mM EPPS pH 8.5

10 mM	EDTA pH 8.0
0.5 %	Triton X-100
3.75 M	NaCl

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CLAIMS

1. A collection of labelled target DNA molecules which are exonuclease derivatives of cDNA.

2. A collection according to claim 1, wherein the cDNA is globally amplified cDNA.

3. A collection according to claim 1 or claim 2, wherein the DNA molecules are labelled by incorporation of labelled nucleotides.

4. A collection according to any preceding claim, wherein the DNA molecules are fluorescently labelled.

5. A collection according to any preceding claim, wherein the labelled target DNA molecules are prepared from cDNA by a complexity reduction technique.

6. A collection according to claim 5, wherein the complexity reduction technique comprises a restriction digestion technique.

7. A collection according to claim 5, wherein the complexity reduction technique comprises a subtraction technique.

8. A collection according to claim 5, wherein the complexity reduction technique comprises a cDNA display technique.

9. A method of producing a collection of labelled target DNA molecules according to claim 1, comprising:

(i) subjecting cDNA, or a derivative thereof, to exonuclease digestion to produce a collection of essentially single-stranded DNA molecules; and

(ii) labelling the single-stranded molecules.

10. A method according to claim 9, wherein the single-stranded molecules are labelled by the action of terminal transferase in the presence of labelled nucleotides.

11. A method of producing a collection of labelled target DNA molecules according to claim 1, comprising:

(i) treating double-stranded cDNA, or a derivative thereof, to obtain a labelled double-stranded DNA population; and

(ii) effecting exonuclease digestion of the labelled population to produce a collection of essentially single-stranded labelled DNA molecules.

12. A method according to claim 11, wherein the labelled double-stranded DNA population is prepared by the action of terminal transferase in the presence of labelled nucleotides.

13. A method according to claim 11, wherein the labelled double-stranded DNA population is prepared by PCR in the presence of labelled nucleotides.

14. A kit for the preparation of a collection of labelled target DNA molecules according to claim 1, the kit comprising:

(i) an exonuclease;

(ii) terminal transferase; and

(iii) labelled nucleotides.

15. A kit for the preparation of a collection of labelled target DNA molecules according to claim 1, the kit comprising:

(i) an exonuclease;

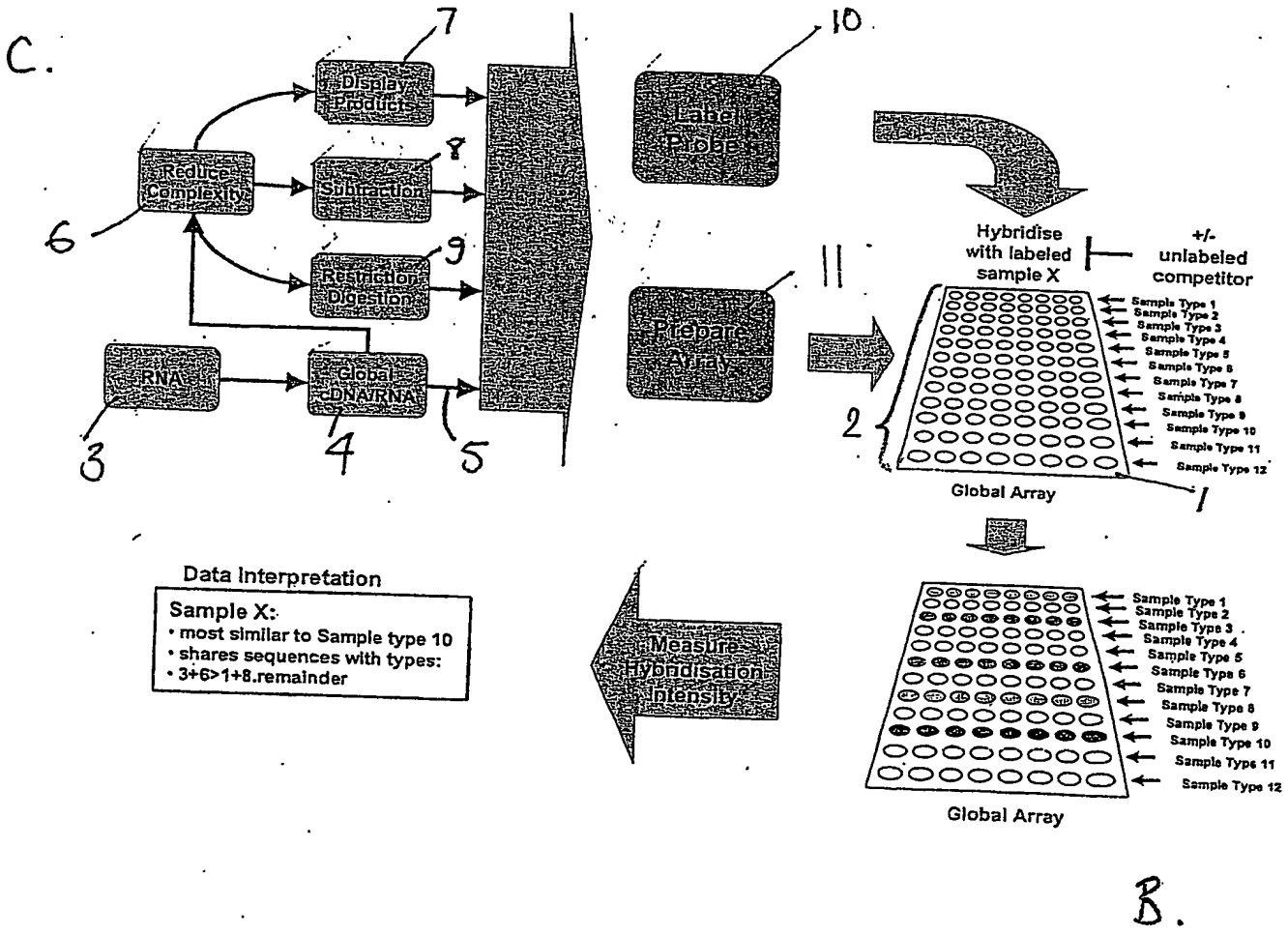
(ii) primers; and

(iii) labelled nucleotides.

16. A kit according to claim 15, further comprising reagents for PCR.

17. A kit according to any one of claims 14 to 16, wherein the labelled nucleotides are fluorescently labelled.

Figure 1.


C.
7
Display Products
8
Subtraction
9
Restriction Digestion
6
Reduce Complexity
RNA
Global cDNA/RNA
3
4
5
10
Label Probe
Prepare Array
11
Hybridise with labeled sample X
+/- unlabeled competitor
Sample Type 1
Sample Type 2
Sample Type 3
Sample Type 4
Sample Type 5
Sample Type 6
Sample Type 7
Sample Type 8
Sample Type 9
Sample Type 10
Sample Type 11
Sample Type 12
2
Global Array
1
A.
Data Interpretation
Sample X:
• most similar to Sample type 10
• shares sequences with types:
• 3+6>1+8.remainder
Measure Hybridisation Intensity
Sample Type 1
Sample Type 2
Sample Type 3
Sample Type 4
Sample Type 5
Sample Type 6
Sample Type 7
Sample Type 8
Sample Type 9
Sample Type 10
Sample Type 11
Sample Type 12
Global Array
B.